Biochimica et Biophysica Acta, 524 (1978) 474—483

BBA 68460

ACTIVATION OF ADENYLATE CYCLASE IN BOVINE ADRENAL CORTEX MEMBRANES BY MAGNESIUM IONS, GUANINE NUCLEOTIDES AND CORTICOTROPIN

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(Received November 11th, 1977)
(Revised manuscript received February 13th, 1978)

Summary

(1) Adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) activity in plasma membranes from bovine adrenal cortex was labile, but was stabilised in the presence of Mg^{2+} or 5′-guanylylimidodiphosphate (Gpp(NH)p). The activation of the enzyme by the nucleotide analogue could not be reversed by washing Gpp(NH)p-pretreated membranes. Similarly, Gpp(NH)p-activation was blocked, but not reversed, by EDTA.

(2) Maximal activation of adenylate cyclase by saturating concentrations of Gpp(NH)p required 20—30 min at 30° C. Once the activation was complete the enzyme was only minimally stimulated by corticotropin. Conversely, in the presence of both hormone and Gpp(NH)p, at 30° C, a maximum activity was attained in under 5 min.

(3) At 20° C, incubation of adrenal membranes for 40—60 min with Mg^{2+} alone, enhanced adenylate cyclase activity 2.4-fold. Gpp(NH)p-activation was not apparent until after 20 min and required about 2 h for completion. Corticotropin increased the initial rate of Gpp(NH)p-activation by at least 5 times and in the presence of both effectors, activity was maximal with 1 h.

(4) The maximum activity elicited by a combination of corticotropin and Gpp(NH)p was 55% greater than that observed in the presence of Gpp(NH)p alone, at both 20 and 30° C.

(5) It is concluded that guanine nucleotides, corticotropin and Mg^{2+} engender new conformational states of adrenal adenylate cyclase which have increased catalytic activity and increased stability to inactivation. An early stage of the guanine nucleotide activation process, possibly the binding, appears

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to require a minimum level of divalent cation. Whilst corticotropin increased the extent of guanine nucleotide activation the major action of the hormone is to enhance the rate of the activation.

Introduction

As a result of studies on membrane preparations from various eukaryotic cells it has become clear that adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) activity may be modulated by hormones, guanine nucleotides and Mg^{2+} [1]. Phosphohydrolase-resistant analogues of GTP such as 5′-guanylylimidodiphosphate (Gpp(NH)p) are potent activators of the enzyme from many cell types [2]. Gpp(NH)p appears to act at the same site as the putative natural effector, GTP, but, in contrast to the native nucleotide, activates the enzyme by a slow process which cannot be reversed easily [3—5]. These observations have led to the suggestion that hydrolysis of GTP may be required to terminate its stimulation of adenylate cyclase activity, by facilitating its dissociation from a nucleotide regulatory site [6,7].

The substrate for adenylate cyclase is $MgATP^{2-}$; however, the enzyme is increasingly activated by Mg^{2+} concentrations in excess of those required to convert the great majority of ATP to $MgATP^{2-}$ [8]. This stimulatory action has been attributed either to a direct activation by the cation [9,10] or to an indirect effect by lowering the concentration of uncomplexed forms of ATP (ATP^{4-}, $HATP^{3-}$) which may be potent competitive inhibitors of the enzyme [4,11].

We have previously shown that corticotropin appears to reduce the Mg^{2+} requirement of adenylate cyclase in a plasma membrane preparation from bovine adrenal cortex [12]. Other workers have demonstrated enhancement of adrenal adenylate cyclase activity by GTP and Gpp(NH)p [13,14]. However, in view of the slow and quasi-irreversible nature of Gpp(NH)p activation it is clear that this process must be studied kinetically in order to obtain meaningful data. Thus, in the present report we have examined the kinetics of adrenal adenylate cyclase activation by Gpp(NH)p and the influence of corticotropin and Mg^{2+} on this process. It is concluded that Mg^{2+} itself, in the absence of any interactions with exogenously added purine nucleotides, is an activator of the adrenal enzyme, and that a major effect of corticotropin is to enhance the rate of guanine nucleotide activation.

Materials

[α-^{32}P]ATP (10-12 Ci/mmol; sodium salt) and cyclic[8-^{3}H]AMP (27 Ci/mmol; ammonium salt) were purchased from the Radiochemical Centre, Amersham, Bucks. The corticotropin used in these studies was Synacthen (corticotropin-(1-24)-tetracosapeptide; $^{1-24}$ACTH) which was generously provided by CIBA Laboratories, Horsham, U.K. 5′-Guanylylimidodiphosphate (disodium salt; Gpp(NH)p) synthesised by ICN, was a generous gift from Dr. S. Howell, University of Sussex. The sources of other materials, the methods for preparation of a plasma membrane fraction from bovine adrenal cortex and for

determination of membrane protein concentration have been documented previously [12]. Medium A consisted of 10 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol.

Methods

Preincubation systems

Method A. Adrenal membranes (0.3—0.6 mg protein) were incubated (1 ml; 30°C) in 25 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol with the effectors, for the time noted in the figure legends. Preincubations were terminated by addition of 10 ml ice-cold medium A and centrifugation (80 000 × *g*; 10 min; 4°C). The pellets were resuspended in 1 ml medium A using a close-fitting glass pestle in the centrifuge tubes (Beckmann; nitrocellulose; 13 ml) and a further 10 ml medium A was added. After shaking and centrifugation as above, the pellet was rinsed three times with medium A and the pellet resuspended in 0.3—0.6 ml medium A. The adenylate cyclase activity of 20 µl aliquots of the washed membranes was determined.

Method B. Adrenal membranes were incubated (30 µl; 30° C) in 25 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol, with the effectors, and for the times noted in the figure legends. Adenylate cyclase assays were initiated by addition of 20 µl assay cocktail to complete the composition of the reaction mixture described below. Modifications to this method are described in the appropriate figures. This method was more convenient than Method A for large numbers of experimental samples

Adenylate cyclase assay

Adrenal membranes were incubated in 25 mM Tris · HCl (pH 7.6) containing [α-^{32}P]ATP (0.6—1.2 µCi), cyclic[^{3}H]AMP (approx. 0.025 µCi), 1 mM cyclic AMP, 0.1 mM ATP, 5 mM $MgCl_2$, 0.2% human serum albumin, 1 mM dithiothreitol, 2 mM phosphocreatine and 25 units/ml creatine kinase at 30° C (unless stated otherwise) in a total volume of 50 µl. The concentrations of other agents and of membrane protein used are indicated in the figure legends. Except where stated otherwise, reactions were initiated by addition of membranes and terminated by addition of 100 µl 'stop-solution' (2% SDS containing 40 mM ATP and 12.5 mM cyclic AMP, pH 7.5). Cyclic[^{32}P]AMP was isolated by the method of Salomon et al. [15] with the modifications described previously [12]. Experimental points represent the means of duplicate determinations which agreed to within less than 7%. All experiments have been performed at least twice with essentially identical results.

Results

When Gpp(NH)p was added directly to adrenal adenylate cyclase assays a steady-state activity was not observed until after 5—10 min incubation (unpublished data). Such initial lag-phases have been reported for other adenylate cyclases assayed under analogous conditions [3,16]. Thus, adrenal membranes were routinely preincubated with guanine nucleotides and other effectors prior to adenylate cyclase assay.

The presence of $MgCl_2$ in preincubations, both in the presence and absence of Gpp(NH)p, enhanced the activity of adenylate cyclase determined subsequently (Table I). $MgCl_2$ (5 mM) was thereafter routinely included in preincubation media. Basal adenylate cyclase activity was not significantly affected by the presence of 1 mM EDTA in the preincubation, but activation by Gpp(NH)p was inhibited. The extent of the inhibition produced by EDTA varied somewhat with the batch of membranes used, as did the adenylate cyclase activities themselves. For example, in the experiment described in Table II, 1 mM EDTA reduced Gpp(NH)p-induced activity only 50%. This variation may reflect differences in the divalent cation content of different batches of adrenal membranes. However, a consistent finding was that EDTA did not significantly reduce the activity of membranes which had been previously incubated with Gpp(NH)p (Table II). This observation indicates that the chelator blocks, but does not reverse, Gpp(NH)p-activation of adrenal adenylate cyclase.

The activated state of adenylate cyclase, produced by preincubation of adrenal membranes with Gpp(NH)p was not reversed by thorough washing of the membranes. Maximal activation of adrenal adenylate cyclase by Gpp(NH)p (as assessed by the inability of the analogue to elicit a further increase in activity, when subsequently added to the enzyme assay) required 30—40 min incubation (Fig. 1). In the absence of Gpp(NH)p, adenylate cyclase activity declined throughout the preincubation, although the stimulation produced by Gpp(NH)p in the subsequent cyclase assay remained essentially constant (1.5—1.6-fold).

The lability of adenylate cyclase activity has long been recognised [8]. In a study of the kinetics of a prolonged process such as Gpp(NH)p-activation, denaturation of the enzyme may complicate interpretation of the data, particularly if different effectors confer different degrees of stability. The adrenal enzyme loses 12% and 45% of its original activity within 40 min at 20 and 30°C respectively in Tris · HCl (pH 7.6)/1 mM dithiothreitol (unpublished data). The enhancement of adrenal adenylate cyclase activity obtained by including Mg^{2+} in preincubation media (Table I) may result from a stabilisation

TABLE I

EFFECT OF Mg^{2+} ON Gpp(NH)p-ACTIVATION OF ADRENAL ADENYLATE CYCLASE

Adrenal membranes (1.35 mg/ml) were incubated (100 µl; 30°C) in 25 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol, in the absence (a) or presence (b) of 0.1 mM Gpp(NH)p. After 30 min incubation, triplicate aliquots (20 µl) were pipetted into tubes containing 30 µl of reagents required for the adenylate cyclase (5 min). In all cases the final concentrations in the assay mixture were 0.04 mM Gpp(NH)p and 5 mM $MgCl_2$ (5 mM in excess of EDTA in the case of EDTA-pretreated membranes). The mean ± S.E.M. of triplicate determinations are indicated.

Preincubation additions	Adenylate cyclase activity (pmol cyclic AMP/5 min per mg protein)	
	(a) —Gpp(NH)p	(b) +Gpp(NH)p
None	206 ± 18	409 ± 21
1 mM $MgCl_2$	259 ± 7	723 ± 13
5 mM $MgCl_2$	314 ± 3	726 ± 24
12.5 mM $MgCl_2$	397 ± 2	895 ± 92
1 mM EDTA	237 ± 8	227 ± 9

TABLE II

FAILURE OF EDTA TO REVERSE Gpp(NH)p-ACTIVATION OF ADRENAL ADENYLATE CYCLASE

Adrenal membranes were subjected to two sequential preincubations before the adenylate cyclase assay. Adrenal membranes were incubated at 30°C in 25 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol and the additions (1) noted in the Table. After 30 min the incubation was terminated and the membranes were washed as described in Method A. The washed membranes were incubated at 30°C in the medium described above, with additions (2) noted in the Table, and after 30 min were washed again as described in Method A. The adenylate cyclase activity of membranes from the second preincubation was determined. Membrane protein in the four sets of membranes ranged from 12 to 14 μg per assay. Adenylate cyclase activity is expressed as pmol cyclic AMP produced per 5 min per mg protein and data represent the mean ± S.E.M. of triplicate determinations. When present, the concentrations of Gpp(NH)p and EDTA were 0.1 and 1.0 mM respectively.

Preincubation additions		Adenylate cyclase activity
(1)	(2)	
None	None	39 ± 1
Gpp(NH)p	None	133 ± 7
Gpp(NH)p + EDTA	None	62 ± 2
Gpp(NH)p	EDTA	116 ± 5

of the enzyme by the cation during the preincubation. The adenylate cyclase activity of membranes which had been preincubated with Gpp(NH)p and then washed to remove unbound nucleotide, appeared more stable than that of

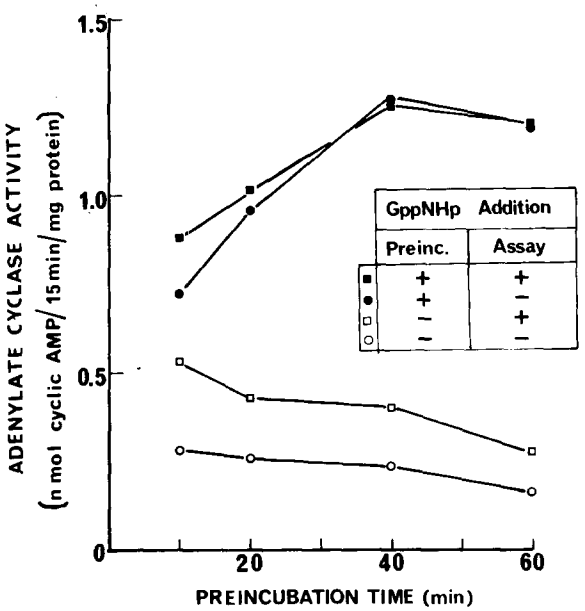

Fig. 1. Time course of Gpp(NH)p-activation of adrenal adenylate cyclase. Adrenal membranes were preincubated at 30°C in 25 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol and 5 mM $MgCl_2$ in the absence (open symbols) or presence (closed symbols) of 0.1 mM Gpp(NH)p. At the indicated times, incubations were stopped and membranes washed as described in Method A. The adenylate cyclase activity of 20 μl aliquots of the washed membranes was determined in the presence (■, □) or absence (●, ○) of 0.1 mM Gpp(NH)p. Membrane protein in the eight sets of washed membranes was determined individually (in triplicate) and ranged from 11.5 to 15.5 μg/assay.

control membranes. The degree of stabilisation appeared to be related to the duration of the pretreatment with the analogue (Fig. 2).

When the concentration of Gpp(NH)p in the preincubation was raised from 0.1 mM (cf. Figs. 1 and 4) to 0.83 mM and 1.67 mM maximal activation of adenylate cyclase still required 20—30 min (unpublished data).

Linear kinetics were observed (Fig. 3) for the adenylate cyclase activity of membranes which had been preincubated (40 min; 30° C) in the presence or absence of Gpp(NH)p, and were assayed subsequently in the presence or absence of $^{1-24}$ACTH. The stimulation by $^{1-24}$ACTH was much greater in control membranes (114%) than in Gpp(NH)p-pretreated membranes (16%). A similar marginal enhancement of adenylate cyclase activity by $^{1-24}$ACTH was observed when membranes, pretreated with Gpp(NH)p for 40 min, were assayed in the presence of 0.5 mM (rather than 5 mM) $MgCl_2$ (unpublished data).

In the presence of both 0.1 mM Gpp(NH)p and 1 μM $^{1-24}$ACTH, maximal activation was attained within the period used for adenylate cyclase assay (5 min), and this activity remained constant over 40 min (Fig. 4). In the absence of hormone, activation by Gpp(NH)p alone took about 20 min to reach a maximum, which was 65% of that observed in the presence of both effectors.

The kinetics of this process were also studied at 20° C (Fig. 5) in order to determine more precisely the effect of $^{1-24}$ACTH on both the rate, and the final extent of Gpp(NH)p activation of adrenal adenylate cyclase and to reduce the potential for enzyme denaturation. Under these conditions the activity of

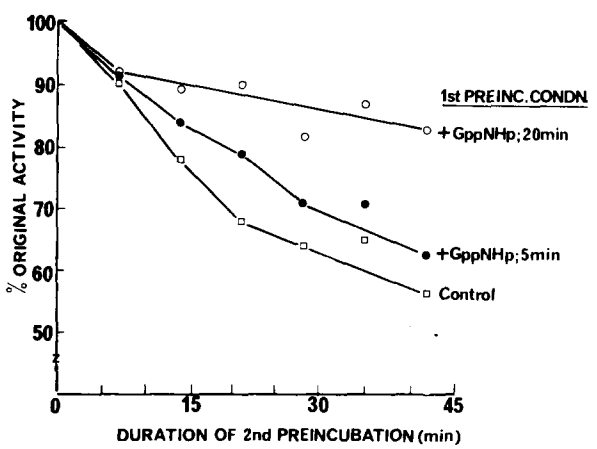

Fig. 2. Gpp(NH)p-pretreatment and the stability of adrenal adenylate cyclase. Adrenal membranes were subjected to two sequential preincubations before the adenylate cyclase assay. 1st Preincubation: adrenal membranes (0.47 mg/ml) were incubated at 30° C in 1 ml 25 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol, 5 mM $MgCl_2$, 0.2% human serum albumin and 0.1 mM Gpp(NH)p for either 5 min (●) or 20 min (○). Control membranes (□) were kept at 4° C in the same medium but in the absence of Gpp(NH)p. The three sets of membranes were washed as described in Method A. 2nd Preincubation: aliquots of the washed membranes (○, 8 μg; ●, 9 μg; □, 10 μg protein) were incubated at 30° C in 30 μl 6.7 mM Tris · HCl (pH 7.6)/0.67 mM dithiothreitol and 0.33% human serum albumin. At the times indicated adenylate cyclase assays were initiated as described in Method B. Results are expressed as the percentage of initial (zero-time) activities. Zero-time activities, obtained after washed membranes had been prewarmed (30° C; 1.5 min) were: ○, 1.10; ●, 0.50; □, 0.20 nmol cyclic AMP/5 min/mg protein.

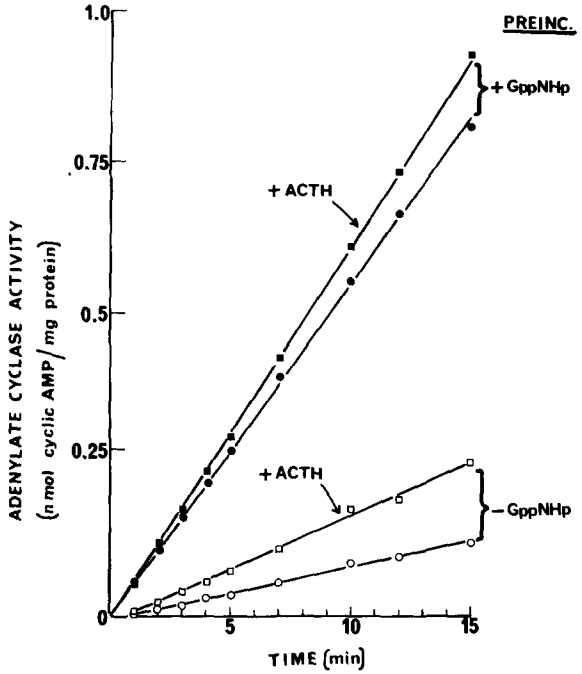

Fig. 3. Effect of Gpp(NH)p-pretreatment on the time course of basal and $^{1-24}$ACTH-stimulated adenylate cyclase activity. Adrenal membranes (0.39 mg/ml) were incubated (40 min; 30° C) in 25 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol and 5 mM $MgCl_2$ in the absence (open symbols) and presence (closed symbols) of 0.1 mM Gpp(NH)p. After washing (Method A), the adenylate cyclase activity of 200-µl aliquots of the membranes was determined. The assay was initiated by addition of prewarmed membranes (4 min; 30° C) to similarly prewarmed reaction mixture (300 µl). Final concentrations were: membrane protein 0.185 mg/ml; $^{1-24}$ACTH, 1 µM when present (■, □). At the times indicated, 50-µl aliquots were pipetted into 100 µl 'stopping-solution' for subsequent isolation of cyclic[^{32}P]AMP. Points represent individual determinations.

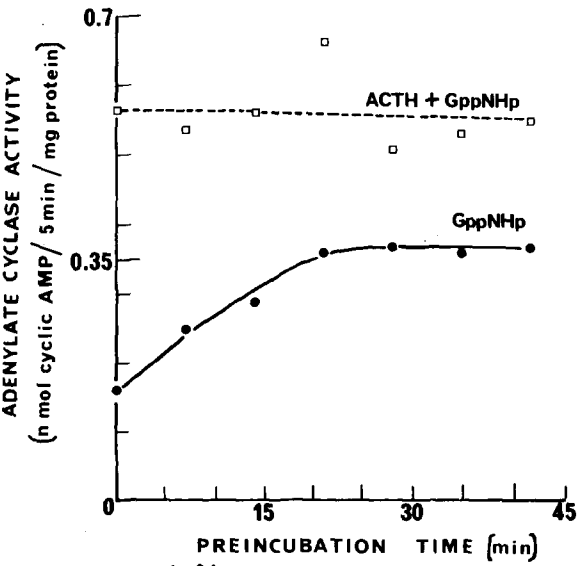

Fig. 4. Effect of $^{1-24}$ACTH on Gpp(NH)p-activation of adrenal adenylate cyclase. Adrenal membranes (21 µg protein) were incubated (30° C, 30 µl) in Tris · HCl (pH 7.6)/dithiothreitol, $MgCl_2$, human serum albumin and Gpp(NH)p in the absence (●) or presence (□) of $^{1-24}$ACTH. The concentrations of the above agents in the preincubation were 1.67 times those in the final adenylate cyclase assay. At the indicated times, the last was initiated as described in Method B. The reaction contained 0.1 mM Gpp(NH)p and (when present) 1 µM $^{1-24}$ACTH and was terminated after 5 min.

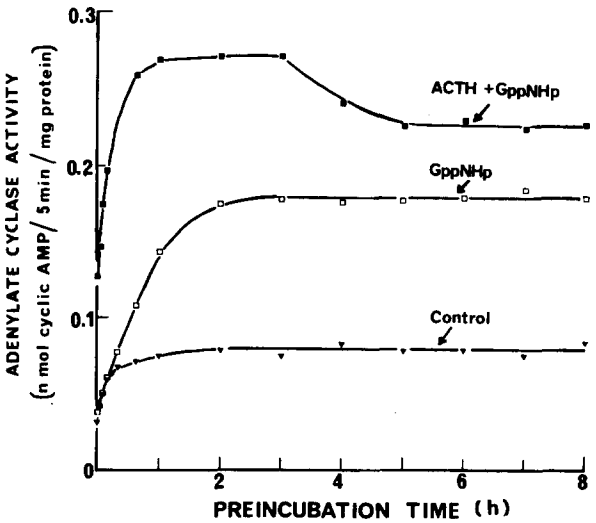

Fig. 5. Activation of adrenal adenylate cyclase at 20°C. Adrenal membranes were incubated (20°C; 0.8 ml) in 25 mM Tris · HCl (pH 7.6)/1 mM dithiothreitol, 0.2% human serum albumin and 5 mM $MgCl_2$. Concentrations of effectors in the preincubation were: □, 0.1 mM Gpp(NH)p; ■, 1 μM $^{1-24}$ACTH + 0.1 mM Gpp(NH)p; ▼, control. At the times indicated, duplicate 20-μl aliquots were pipetted into a 30-μl solution containing the components required to complete the composition of the adenylate cyclase assay. Assay time was 5 min at 20°C. Zero-time points contained 0.04 mM Gpp(NH)p (□), or 0.04 mM Gpp(NH)p + 0.4 μM $^{1-24}$ACTH (■). Membrane protein was 20.5 μg per assay.

membranes incubated with Mg^{2+} alone, increased over 1 h to a stable level of activity, 2.4-fold greater than that at zero time. This initial increase in activity may reflect equilibration of the enzyme with Mg^{2+}. It is possible that Gpp(NH)p-activation, which in the absence of hormone was not apparent until after 20 min at 20°C, is dependent on the Mg^{2+}-dependent process (cf. Table I). The maximum activity, attained after 1 h, in the presence of both $^{1-24}$ACTH and Gpp(NH)p was 55% greater than that observed, after 2 h, in the presence of the nucleotide analogue alone (i.e., the same increment as observed at 30°C; Fig. 4). After 3 h the activity in the presence of hormone and Gpp(NH)p declined by 18% to reach a new stable level after 5 h.

Discussion

The resistance of the Gpp(NH)p-activated state of adenylate cyclase to reversal to a basal state is well documented (e.g. refs. 3 and 5). It has been suggested that the analogue acts in an irreversible process which may involve covalent bond formation with some component of the enzyme [5,17]. However, Sevilla and Levitzki [18] have shown that the activated state of turkey erythrocyte adenylate cyclase, produced by pretreatment of the membranes with Gpp(NH)p and epinephrine, can be reversed slowly by subsequent incubation with the catecholamine and a purine nucleotide. Thus, although bound Gpp(NH)p may dissociate only very slowly it appears to be exchangeable with free purine nucleotide. This finding may also explain the decline in activity of the maximally-activated adrenal enzyme after 3 h at 20°C, since in this experi-

ment (Fig. 5) the enzyme and corticotropin were transferred simultaneously to a medium which contained ATP.

At saturating concentrations of Gpp(NH)p maximal activation of adrenal adenylate cyclase required 20—30 min at 30° C. This slow activation process which, on completion, renders the enzyme insensitive to hormonal stimulation (Fig. 3) has been reported for other adenylate cyclase systems [16,17]. The maximal increment in the extent of Gpp(NH)p-activation observed in the presence of corticotropin was only 55%. By contrast, the major effect of the hormone was to markedly accelerate the rate of activation of the enzyme by Gpp(NH)p. At 20° C, corticotropin enhanced the initial rate of activation by at least 5 times (Fig. 5).

These observations lend support to models for the regulation of adenylate cyclase which are based mainly on studies on the rat hepatic [4] and turkey erythrocyte enzyme [6,7]. These models propose that guanine nucleotides are the primary activators of adenylate cyclase and that hormones act by accelerating a conformational change in the enzyme subsequent to the binding of the nucleotide.

The finding that EDTA blocks, but does not reverse, Gpp(NH)p activation of adenylate cyclase (Table II) has also been noted for the turkey erythrocyte enzyme by Spiegel et al. [19]. These authors showed that EDTA inhibited both adenylate cyclase activation and [^{3}H]Gpp(NH)p binding when included in preincubation media. However, washing Gpp(NH)p-pretreated membranes in EDTA solutions did not cause the cyclase activity to diminish to basal values. These observations suggest that a minimum level of divalent cation may be required for binding to the nucleotide site, but that once Gpp(NH)p is bound to this site, then either the activation process becomes independent of divalent cation or the nucleotide site becomes inaccessible to EDTA.

We have reported previously that corticotropin and GTP appear to act synergistically to reduce the requirement of adrenal adenylate cyclase activity for Mg^{2+} [12], possibly by raising the affinity of the system for this cation. At that time it was not possible to state whether Mg^{2+} activated the enzyme either indirectly, by lowering the concentration of trace amounts of uncomplexed ATP or directly by its intrinsic properties as a positive effector of the system. However, the data in Fig. 5 strongly suggest that Mg^{2+}, in the absence of any direct interactions with exogenously added purine nucleotides, is an activator of adrenocortical adenylate cyclase. This is the first demonstration of a specific stimulatory effect of the cation, per se, on the adrenal enzyme.

In summary, adrenocortical adenylate cyclase is activated by corticotropin, guanine nucleotides and Mg^{2+}. Under in vitro conditions, Mg^{2+} and Gpp(NH)p appear to stabilise the enzyme against denaturation. It is possible that, in vivo, a steady-state level of GTP may be bound at the nucleotide site. In this situation corticotropin may accelerate a conformational change in the cyclase system, which in the absence of hormone, proceeds at a very slow rate and is dependent on the presence of GTP at the nucleotide site. One of the effects of this conformational change may be to raise the affinity of a divalent cation binding site for Mg^{2+} with the consequence that the catalytic activity of the system is increased.

Acknowledgements

Financial support for this work from the Medical Research Council is gratefully acknowledged, as is tenure of a Science Research Council postgraduate studentship (for PG) and an EMBO long-term Fellowship (for DS).

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